IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Bonnert	Art Unit	: 1624
Serial No.	: 10/528,316	Examiner	: Susanna Moore
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Title	: NOVEL COMPOUND		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION BY ROGER VICTOR BONNERT

I, Roger Victor Bonnert do hereby declare as follows:

That

1. I am an Associate Director in Medicinal Chemistry at AstraZeneca, R&D Charnwood, Bakewell Road, Loughborough, Leics, LE11 5RH, UK.
2. I have a BSc Hons in chemistry from the University of Leicester, and a PhD in synthetic organic chemistry also from the University of Leicester, UK. I undertook two years post-doctorate study, firstly at Indiana University, USA and secondly at the University of Texas, USA.
3. I have 17 years experience in the pharmaceutical industry based at Charnwood, firstly with Fisons plc, then Astra AB and now with AstraZeneca.
4. I was a member of a project team at Charnwood from January 1999 working in the area of CXCR2 antagonists (hereinafter referred to as "the team").
5. I am the inventor named in Patent Cooperation Treaty patent application WO2004/026880 and corresponding worldwide patent applications, including U.S.S.N. 10/528,316.
6. The invention in WO2004/026880 is based on the discovery that a compound falling within the scope of published patent application WO2001/25242 (AstraZeneca) has an improved pharmacological profile when compared with what I believe are the structurally most similar published compounds (Examples 4 and 7 of WO2001/25242).
7. It is desirable for a CXCR2 antagonist that will be administered as an oral drug to have a suitable balance of *in vitro* (protein free) potency and % free fraction in blood. It is only the free fraction that is available to bind to the CXCR2 receptor. The free fraction in

blood is controlled principally by plasma protein binding and as such the Whole Blood Potency (WBP) can be derived from a combination of *in vitro* potency and plasma protein binding. WBP can be calculated by the following formula: WBP (IC_{50}) = *in vitro* potency (IC_{50}) x 100% free fraction.

8. The team wanted to find a CXCR2 antagonist that has a whole blood pIC_{50} of greater than 6 and a bioavailability of greater than 20%. It was believed that this combination of properties would provide an oral drug that would be effective at a relatively low dose and also would minimize the consequence of intersubject variability. A suitable surrogate for human bioavailability can be measured in the rat (Chious W. L. & Barve A., Pharm Res 15:11 1998, 1792-1795).

9. Pharmacological profiling of the compounds in WO2004/026880, and the compounds of Examples 4 and 7 of WO2001/25242, was performed according to the protocols set out in Annexes 1, 2 and 3 to this declaration. The full results for these compounds are shown in Annex 4.

10. It can be seen from Annex 4 that the compound tested with the best balance of properties as a CXCR2 antagonist when administered as an oral drug is Example 2 of WO2004/026880, i.e., 5-[[(2,3-difluorophenyl)methyl]thio]-7-[[2-hydroxy-1(hydroxymethyl)-1-methylethyl]amino]-thiazolo[4,5-*d*]pyrimidin-2(3*H*)-one, monosodium salt. This is the only compound to have a bioavailability of greater than 20% and a whole blood potency pIC_{50} of greater than 6.

11. While Example 4 of WO2001/25242 has a high bioavailability (57%) and an *in vitro* potency of pIC_{50} = 8.7, it has a low free fraction (0.15%) due to high plasma protein binding. This reduces the free concentration in blood, leading to a calculated whole blood potency of pIC_{50} = 5.9

12. Example 7 of WO2001/25242 has a good *in vitro* potency of pIC_{50} = 8.3 and slightly reduced plasma protein binding (0.76% free) when compared to Example 4, giving a calculated whole blood potency of pIC_{50} = 6.2. But it has low bioavailability (11%).

13. Example 1 of WO2004/026880 has an *in vivo* potency of pIC_{50} = 8.2 and plasma protein binding of 0.99% free, giving a calculated whole blood potency of pIC_{50} = 6.2. It has a bioavailability of only 7%.

14. Example 2 of WO-2004/026880 has an *in vivo* potency of $pIC_{50} = 8.2$ and protein binding (1.55% free), giving a calculated whole blood potency of $pIC_{50} = 6.4$. It also has a bioavailability of at least 22%.

15. In light of the unexpected enhancement in bioavailability seen with Example 2 of WO2004/026880, the team also prepared the analogous sodium salt of Example 7 of WO2001/25242 to determine if the improvement in bioavailability was general phenomenon, but this was found not to be the case. (2, 3, 3% individual results for rat bioavailability mean = 3%, CMC/Tween formulation).

16. Drug discovery is an uncertain process. I believe that the person of ordinary skill in the art, having regard to the pharmacological profile of the compounds of Examples 4 and 7 of WO2001/25242, and wishing to make further useful CXCR2 antagonists would not have been able to predict what structural changes to make to those compounds in order to obtain a compound having the pharmacological profile of the compound described above.

17. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

_____ _____

Dated Roger Victor Bonnert

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

ANNEX 1

Method for determination of CXCR2 potency

The potency of antagonist at the human CXCR2 receptor was determined *in vitro* by quantifying their ability to inhibit specific binding of the CXCR2 radioligand, [^{125}I] interleukin-8 (IL-8), from membranes of HEK293 cells transfected with the human recombinant CXCR2 receptor.

Experimental procedure

Materials

Commercially sourced materials were obtained as follows:
U-bottomed 96-well plates (3799) and 225 cm^2 vented cap culture flasks (3001) from Costar, Corning, Kent UK. Multiscreen filter plates (0.45 μm; MAHV N45 50), vacuum manifold and pump (XF54 230 50) from Millipore, Watford, UK. N-[2-hydroxyethyl]piperazine-N'-[2ethaneusulphonic acid] (HEPES; H-3375), gelatin (G9382), dithiothreitol (DTT;D06052), sodium chloride (S3160/63), sodium hydroxide (B6506), bacitracin (B0125), inactivated foetal calf serum (FCS; CR0848) and DMSO Fluka Chemika (41648) from Sigma, Poole, UK. MicroScint-O (6013611) Packard Bioscience, Pangbourne, UK. Complete protease inhibitor cocktail tablets (1836145) from Boehringer Mannheim, GmbH, Germany, Human recombinant [^{125}I]IL-8 74 TBq/mmol, 0712 MBQ/ml (IM249) from Amersham Horsham UK. All other tissue culture reagants were purchased form Invitorgen, Paisley, Scotland, UK. All other chemicals reagents were analytical grade from Fisher Scientific, Loughborough, UK

Solutions:

HEPES-buffered slat solution pH 7.4 containing HEPES (10 mM), potassium chloride (2.7 mM), sodium chloride (137 mM), potassium hydrogen phosphate (0.4 mM), calcium chloride 1.8 mM), magnesium chloride (1 mM), gelatin (0.1% (w/v)) and bacitracin (100 μg/ml). HEPES-buffered Tyrodes's solution pH 7.4 containing HEPES (10 mM), potassium chloride (2.7 mM), sodium chloride (137 mM), potassium hydrogen phosphate (0.4 mM) glucose (11 mM).

Hypotonic buffer: 3:1 mix of water: HEPES-buffered Tyrode's solution.

Cell Culture and membrane preparation

HEK293 cells were transfected with human CXCR2 (EMBL L19593) CDNA, previously cloned winto the eukaryotic expression vector RcCMV. Cloned cell-lines were generated from stably-transfected geneticin-resistant populations. Cells were routinely grown to approximately 80% confluence in DMEM medium containing 10% (v/V) foetal calf serum and glutamine (2 mM) in a humidified incubator at 37°C , 5% CO_2. Cells were harvested from flask using Accutase ™ at 37°C for 3 to 5 minutes and resuspended on ice in hypotonic buffer at a density of $2x10^7$ cells/mL. Membranes were prepared on ice by homogenization using polytron tissue homogenizer set at 22000 rpm. The membrane fraction was purified by sucrose gradient centrifugation where homogenized cells were layered onto 41% (w/v) sucrose solution then centrifuged at 140000 *g* for 1 hour at 4°C. The membrane pellet was re-suspended at 1 $X10^8$ cell equivalents/m: in HEPES-buffered Tyrode's solution and subsequently stored in aliquots at -80°C. All buffers used for membrane preparation and storage were made in the presence of 1mM DTT and Complete Protease Inhibitor ™ cocktail tablets, made up to manufacturers instructions.

Assay Protocal

Assays were performed in HEPES-bufferd slat solution in 96-well plates. [^{125}I]IL-8 was used at a final concentration of 0.06 nM, pre-diluted for a 9.6 nM stock. The final DMSO concentration in the assay was 1 5 (v/v). Test compounds were prepared by serial dilution in DMSO followed by ten-fold dilution into HEPES- buffered salt solution to five a working solution containing compound and 10% MSO. The control for total binding (B0) of [^{125}I]IL-8 was determined in the absence of compound, The control for non-specific binding (NSB) was determined by measuring [^{125}I]IL8 binding in the presence of (1*R*)-5-[[(3-chloro-2-fluorophenyl)methyl]thio]-7-[[2-hydroxy-1-mehtylethyl]amino]thiazolo[4,-5*d*]pyrimidin-2(3*H)*-one dehydrate, sodium slat 1μM final concentration. Frozen aliquots of membranes were defrosted and diluted to a concentration previously determined to give approximately 10% binding of total radiolabled added, typically about 1 x 10^6 cell equivalents/mL. The assay copenents

were added to each well as follows; one-tenth volume test compounds or controls in buffer containing 10% DMSO, one-tenth volume radiolabel, eight-tenths volume diluted membranes. The plates were sealed and incubated for 2 hours at room temperature. Following incubation, the assay mixture was filtered then washed with two volumes of cold HEPES-buffered salt solution using a Millipore vacuum manifold. The filtration plate was allowed to air dry then either the individual filters were punched out into polypropylene test tubes and the radioactivity measured by direct gamma counting using a Cobra II Gamma counter (Packard BioScience) for 1 minute per sample of alternatively, the whole filtration plate was placed in a carrier plate and 50μL of MicroScint-O added to each well. 96 well plate scintillation counting was performed using a TopCount instrument (Packard BioScience) for 1 minute per sample well.

Data analysis

Specific binding of [^{125}I]IL-8 was calculated by substrcting the mean of the control NSB values determined in each asay plate. Data was transformed into concentration-response plots and expressed as a percent relative to total specifically bound [^{125}I[IL-8 (B0-NSB). The IC_{50} was defined as a molar concentration of compound required o give 50% inhibition of specifically bound [^{125}I]IL-8. The IC_{50} values were transformed into the reciprocal logarithm (pIC_{50}) for calculation of descriptive statistics (mean$\pm$ SE), The pIC_{50} values approximated to the binding affinity (pKi) since the concentration of [^{125}I]IL-8 used (0.06 nM) was below the Kd (equilibrium dissociation constant) determined for Il-8 (1.2 nM).

ANNEX 2

Measurement of Plasma Protein Binding

Introduction

The extent of binding of a drug to plasma proteins is a crucial factor in determining its *in vivo* potency and pharmacokinetics. The method used for determining the extent of plasma protein binding involves equilibrium dialysis of the compound in the plasma and buffer are then determined using high pressure liquid chromatography (HPLC) with mass spectroscopy (MS) detection. The dialysis method involves the use of mixtures of up to 10 compounds simultaineoulsy. It has baeen shown that at the concentrations used in the assay, there is no significant difference in the results when compounds are run singly or in mixtures.

Method

Membranes (molecular weight cut-off 5000) were first prepared by soaking in the dialysis buffer for a minimum of 1 hour. The dialysis membranes were then mounted into the dialysis cells.

Stock solution sof compounds in dimethylsulphoxide (DMSO) were prepared. This, and all subsequent liquid handling steps, were normally done using a Tecan liquid handling robot. Mixtures of up to five compounds were used. The concentration of each compound in a mixture was normally 1 mM. The mixtures were chosen such that each mixture contains compounds that all have at least a 5 unit differenc ein molecular weight from one anotehr.

Frozen plasma (EDTA anticoagulant) sourced from the global plasma supply at Alderley Park were normally used for the human plasma binding experiment. The pH of the plasma was adjusted to 7.4 using 1 M HC1 immediately before use.

The stock DMSO solution of compounds (7.5 μ) was then added to the dialysis cells along with plasma (750 μl). This was done in dupliate for each mixture. This gave a 1% DMSO in plasma solution with each compound at a concentration of 10 μm (if the stock solution was the standard 1 mM). The dialysis cells were then sealed, secured in a Dianorm rotator unit and equilibrated for 18 hours at 37 °C. While the dialysis cells were being equilibrated, the DMSO stock solutions were used for generating optimised HPLC/MS methods for use in the final analysis of the plasma and buffer samples.

After equilibration, the cells were opened and a Tecan liquid handling robot was used to remove aliquots from the plasma and buffer sides of each of the dialysis cells. Blank plasma was then added to the buffer samples and buffer added to the plasma samples such that each sampole was in a matrix of 6-fold diluted plasma. Standards were then prepared from the DMSO stock soltuions and blank 6-fold diluted plasma. The concentrations of the four standards were normally 50 nM, 150 nM, 500 nM and 2500 nM.

The samples and standards were then analysed using HPLC with MS detection, which allows deconvolution of the mixtures of compounds. The HPLC method involved a forward flushing column switching technique that allows direct injection of the diluted plasma.

Calculation of Results

The chromatograms were porcessed using the MassLynx software that automatically calculates a calibration curve for each compound in a mixture and then interpolates the concentrations of buffer and plasma samples. These concentrations still need corrctions for the dilution of the plasma. The percentage bound was calculated from the MassLynx data using the following equation:

The factor of 1.2 in the numerator accounts for the small dilution of the aqueous samples with plasma. The factor of 6 in the denominator serves to correct for the 6-fold dilution of the plasma samples with buffer.

The % free (100-%bound) for each compound was calculatead from the concentration data, and then recorded.

ANNEX 3

Pharmacokinetics in the Rat

Introduction

This describes the methods used to obtain *in vivo* pharmacokinetic parameters in the male rat. It is applicable for use with any compound but may need modification based on such parameters as solubility, assay sensitivity, anticipated clearance and half-life, when the default formulation, dose level or sampling intervals may be inappropriate. The method described here represents a standard approach from which justified and documented modifications can be made this method also allows for single compounds or mixtures (cassettes) to be administered.

Dose Preparation

A standard dose solution of 1 mg ml^{-1} was prepared. The recommended dose vehicle (if the compound was not sufficiently soluble in isotonic saline) was 50% PEG 400:40% sterile water. The required mass of compound was dissolved in the PEG400 before addition of the water. The concentration of the compound in the dose solution was assayed by diluting an aliquot to a nominal concentration of 50 μg ml^{-1} and calibrating against duplicate injections of a standard solution and a QC standard at this concentration.

Dosing

Compounds were administered intravenously as a bolus into a caudal vein to groups of three 250-350g rats (approximately 1 ml kg^{-1}). Delivered doses were estimated by weight loss.

Food was not usually withdrawn from animals prior to dosing, although this effect can be investigated if necessary.

Sample Collection

Pre-dose samples were taken from the oral group. Blood samples (0.25ml) were taken into 1 ml syringes, transferred to EDTA tubes and plasma was prepared by centrifugation (3 min at 13000rpm) soon after sample collection.

Sampling times (min) for the standard protocols

iv	oral
2	pre
4	20
8	40

15	60
30	120
60	180
120	240
180	300
240	360
300	

Sample Analysis

The concentration of the analyte(s) were determined in plasma quantitative by mass spectrometry.

Preparation of Standards and QCs

Standard and quality control stock solutions were prepared at a concentration 50 µg/ml in methanol. The standards and QC stocks were diluted by the TECAN GENESIS and spiked into plasma according to the following table:

Serial Dilution Program			50 µg/ml stock	
solution	Volume stock (µl)	Volume Diluent (µl)	Std. Conc. (ng/ml)	QC Conc. (ng/ml)
A	90 of initial stock	810	1000	-
B	300 of A	300	500	500
C	300 of B	300	250	-
D	200 of C	300	100	100
E	300 of D	300	50	-
F	300 of E	300	25	-
G	200 of F	300	10	10
H	300 of G	300	5	-

10µl of each of the above solutions A - H produced by serial dilution of the combined standard stock, and 10µl of solutions B, D and G, produced by serial dilution of the combined QC stock,

are added to 96 well 1.2 ml polypropylene tubes containing 50µl blank plasma by the TECAN. The final concentrations of the standard curve and QC samples produced are shown in the table above. Higher or lower ranges can be obtained using a concentrated or dilute initial stock solution.

Preparation of Samples

To each of the test samples, standards and QCs was added 150 µl of water. The samples were arranged in the order defined below:

1. Standards in order of ascending concentration
2. QCs in order of ascending concentration manual standard.
3. Test samples from IV dosed animals (1M, 2M and then 3M samples)
4. QCs in order of ascending concentration
5. Test samples from PO dosed animals (4M, 5M and then 6M samples)
6. QCs in order of ascending concentration.
7. Standards in order of ascending concentration.

The samples were then capped, mixed by repeated inversion and then centrifuged at 3500 rpm in an IEC CENTRA centrifuge for 20 minutes. Aliquots (120 µl) of each sample were analysised LC/MS.

Mass Spectrometry

A TSQ700 or a TSQ or SSQ7000 mass spectrometer with a HP1100 HPLC system was used. The sources used were APCI or ESI. Standard and quality control samples covering the range of concentrations found in the test samples were expected to be within 25% of the nominal concentration.

Results

Pharmacokinetic data analysis and tabulation was achieved using WinNonlin and Excel. A standard non-compartmental analysis was used to estimate the parameters tabulated. Bioavailability was calculated from the ratio of the iv and oral AUC (the integral of the plasma

concentration time curve) once dose normalized. Data was entered onto the company data base. Individual entries were made for each administration to each animal.

ANNEX 4

Pharmacological profiles

W0-2001/25242

Compound	**CXCR2 pIC_{50} Individual results and mean**	**Human plasma Protein Binding % free Individual Results And mean**	**Rat Bioavailability (F%) Individual Results And mean (formulation)**
Example 4	8.8, 8.6, 8.6, 8.7 Mean = 8.7	Free= 0.15	45, 72, 53 Mean = 57 (CMC/Tween)
Example 7	8.4, 8.2 Mean = 8.3	0.71, 0.81 Mean free = 0.76	8, 7, 18 Mean = 11 (CMC/Tween)

WO-2004/026835

Compound	**CXCR2 pIC_{50} Individual Results And mean**	**Human plasma Protein binding % free Individual Results and mean**	**Rat Bioavailability (F%) Individual Results And mean (formulation)**
Example 1	8.4, 8.1, 8.1 7.6, 8.3, 8.3, 8.3, 8.6, 8.6, 7.8, 8.4 Mean=8.2	0.84, 0.74, 1.86, 2.28, 0.61, 0.52, 1.66, 1.8, 0.71, 1.03 Mean free= 1.21	35, 28, 44, Mean =36 (CMC/tween 37, 38, 33 Mean = 36 (PEG/DMA)